A. Specific Aims

Rhamnolipids (RHL) and surface motility are important virulence factors that contribute to the pathogenesis of chronic lung infections caused by *Pseudomonas aeruginosa* (PA) in cystic fibrosis (CF) patients. Surface motility and RHL contribute to pathogenesis by promoting persistent colonization of pulmonary tissue and facilitating the dissemination of bacteria from stressed biofilm communities to a new location. A novel form of surface translocation that occurs in the absence of surface-exposed motility organelles (flagella and type IV pili) was recently discovered in PA and shown to be dependent on rhamnolipids (1). Two types of flagella/pili independent (FPI) motility, active and passive gliding motility, have been described in other bacterial species. Passive gliding motility (PGM) is attributed to the secretion of a surfactant that reduce frictional forces between a bacterium and a surface. Expansive forces produced during cell division and reduced frictional forces result in the lateral spreading of colonies. Active gliding motility (AGM) also depends on glycolipids or glycolipid-like substances; these are responsive to environmental signals and form complex colony patterns on agar surfaces. AGM has been genetically characterized in *Myxococcus xanthus* and *Flavobacterium*, but the molecular engine that powers this type of surface translocation has not been identified for any organism (2). To date, the fundamental mechanisms associated with PA FPI motility (including the secretion of rhamnolipids across the gram-negative cell wall) are not known. This appears to result from the widely held misconception that FPI motility in PA is a result of PGM. This is based on the absence of data to implicate an active driving force. Recent advances in computer-based (*in silico*) prediction analysis of biological factors with unknown function are facilitating the discovery of genes and proteins responsible for novel biological phenomenon (3). By employing an *in silico*-based analysis of proteins that may be involved in rhamnolipid secretion, a novel set of factors has been discovered that have high orthology to components of the gliding motility system (Gld) molecular machinery that drives AGM in other bacterial species. Importantly, a Gld-like ABC transporter system, encoded by three linked genes (PA3670, PA3671, and PA3672) has been identified and the putative proteins encoded by this system are predicted to have high functional orthology to Gld system components that mediate glycolipid secretion in other bacteria.

The long-term goal of this research is to elucidate the molecular mechanisms that underlie PA FPI motility. The objective of this specific proposal is to characterize the role that PA Gld orthologs play in FPI motility and rhamnolipid secretion. **The central hypothesis of the proposed research is that *P. aeruginosa* FPI surface translocation and rhamnolipid secretion is mediated by components of a Gld that is orthologous in multiple bacterial species.** Thus, the experimental focus of the proposed studies is designed to determine the role of Gld components in FPI surface translocation and rhamnolipid secretion with the following **specific aims:**

1. **Identify the involvement of the Gld orthologous genes in FPI surface translocation by mutational analysis of the *gld*-like locus.** Hypothesis: *FPI surface translocation in PA is dependent on the PA Gld-like motility system known to mediate AGM in other bacterial species.*
2. **Determine if expressing the Gld-like ABC transporter system in a recombinant rhamnolipid producing strain of *E. coli* increases the levels of extracellular rhamnolipids (RHL).** Hypothesis: *Expression of the Gld-like ABC transport system in recombinant RHL producing E. coli will increase the efficiency of RHL production.*

The expected outcomes of these aims are: **1)** Confirm that each Gld-like gene encodes a functional protein; **2)** Confirm that these Gld orthologs mediate FPI surface translocation; **3)** Confirm that rhamnolipid secretion is mediated by a Gld-like ABC transport system.

The results from the proposed work are expected to have an important impact on bacterial motility and pathogenesis because they will identify the mechanistic basis for a novel surface motility system and begin to elucidate the currently unknown mechanism of rhamnolipid secretion. Surface motility and rhamnolipid secretion are essential behaviors for successful PA pathogenesis and its persistent colonization of pulmonary tissues. Many of the proteins associated in Gld-mediated surface translocation (including those involved in rhamnolipid secretion) are likely to provide new targets for therapeutic interventions in the treatment of CF/COPD chronic pulmonary *P. aeruginosa* infections.

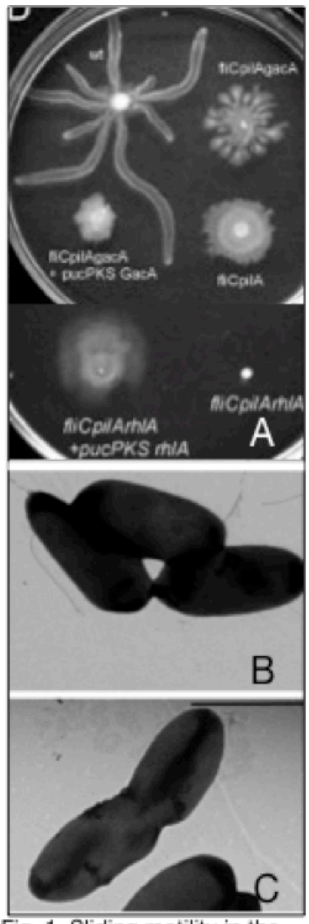

Fig. 1. Sliding motility in the absence of pili and flagella is dependent on rhamnolipid biosynthesis. A) Motility assay of WT P. aeruginosa (top left) fliCpilA (middle right), fliCpilArhlA (bottom right) and rhlA complementation by expression from a plasmid (bottom left). A) Sliding motility in bacteria with deletions mutations in global regulatory genes develops complex patterns. fliCpilAgacA top right and GacA complementation by expression from a plasmid (middle left). TEM images of bacteria expressing or lacking surface exposed motility surfaceB) WT, normal pil and flagella surface expression compared to C) fliCpilA mutants.

Motility and the biofilm lifestyle are linked behaviors critical to *P. aeruginosa* pathogenesis and the maintenance of chronic infections. Pseudomonas aeruginosa is a Gram-negative opportunistic pathogen that causes chronic lung infections in cystic fibrosis (CF) and chronic obstructive pulmonary disease (COPD) patients. A hallmark of *P. aeruginosa* chronic lung infections is the ability of the bacteria to adopt a biofilm lifestyle that confers specific advantages over planktonic growth, including host immunity evasion and enhanced resistance to antimicrobials. The biofilm lifestyle is characterized by a sessile mode of growth of bacterial cells that are attached to a surface and encased in a matrix of extracellular polysaccharides, proteins, and DNA(*1*). There are three main stages in biofilm development: attachment, maturation, and dispersal. The initial attachment stage is well characterized and mediated by surface exposed adhesins, exopolysaccharide (EPS) biosynthesis, motility, and quorum sensing. During the maturation stage cells undergo poorly understood physiological adaptations thought to contribute to persistent infections. The dispersal of biofilm cells in a nutrient deplete environment allows for proliferation into new areas. The majority of biofilm forming bacterial species are thought to disperse through a passive detachment process that is mediated by environmental sheer forces. However, *P. aeruginosa* dispersal is mediated by an active process termed 'seeding dispersal'. This process although poorly understood, involves the differentiation of subpopulations, the formation of mushroom shaped fruiting bodies, and the hollowing of the cap portion of the fruiting body, which then leads to the release of cells capable of swimming motility that promotes dissemination into new environments (*2*).

Seeding dispersal appears to play an important role in chronic *P. aeruginosa* CF and COPD infections. A recent study characterizing the functional traits important in pathogenesis and associated with the dispersal of *P. aeruginosa* biofilm populations found that dispersal-seeding cells exhibited an untypical increase in heterogeneity in all tested traits. The differentiation potential of variant isolates grown in liquid culture was tested to determine if cells had an innate tendency towards differentiation. It was determined that all liquid-gown cells were identical to the parental strain. These data suggested that the biofilm dispersal process might have an influence on the increased diversification of dispersing cells (*3*, *4*). A heterogeneous seeding population is thought in contribute to the ability of *P. aeruginosa* to quickly adapt to the CF lung and maintain a chronic infection by promoting the mutagenesis of biofilm cells. Functional variants that are best fitted for survival in the CF lung environment become the dominant strain (x)(*3*). As seeding dispersal appears to play an important role in chronic *P. aeruginosa* CF infections is it important understand it better.

The types of motility that mediate seeding dispersal are not well understood, but the secretion of rhamnolipids appears to be involved in the process. Motility organelles, particularly flagella, are not typically associated with dispersal fruiting body formation. In support of this idea, a recent 2013 study found that during the initial stages of the biofilm dispersal process, the genes required for flagella-mediated surface motility were decreased in expression, whereas rhamnolipid biosynthesis genes were increased in expression (*2*). The initial stage of the seeding process is characterized by the formation of the 'stalk' of the mushroom shaped fruiting body. Interestingly, non-motile cells lacking type VI pili (*ΔpilA)* are able to produce fruiting body structures capable of dispersing cells, even though they lacked the mushroom-like cap portion. The cap structure formation occurs during later development and is depend on type IV pili. These results suggest that the formation of the stalk occurs by a surface movement powered by expansive forces (*5*). A novel form of surface motility, independent of flagella and type IV pili was recently discovered (*6*) and is the focus of this application. It is likely that this new form of motility is involved the

seeding dispersal process; however, we will not address this possibility here. To date this form of motility has not been characterized beyond the initial report. However, it is known to be dependent on the biosynthesis and secretion of rhamnolipids (*6*).

Flagella-pili independent (FPI) motility: a novel form surface motility dependent on rhamnolipids. In 2008 Murray, et al. (*6*) reported a novel form of surface translocation that occurs in the absence of flagella and type IV pili and termed this behavior 'sliding' motility. This motility will be referred to in this proposal as FPI motility. Previously to this discovery, all known forms of *P. aeruginosa* motility required surface exposed organelles. FPI motility was identified as colony expansion in a non-flagellate/non-piliated *Δ fliC pilA* mutant strain. Rhamnolipids were found to be required for FPI motility by examining the colony expansion phenotypes of a triple mutant strain *Δ fliC pilA rhlA*, that is non-flagellate, non-piliated, and deficient in rhamnolipid biosynthesis and importantly found to be deficient in FPI motility. Complementation of the rhamnolipid biosynthesis deficiency with a wild-type *rhlA* gene present on a high-copy plasmid restored FPI motility. This confirmed that the lack of the rhamnolipid biosynthesis gene was responsible for the FPI motility deficient phenotype (*6*). Figure 1 shows representative examples of FPI-motility and the rhamnolipid deficient phenotype described above.

Sliding motility is defined as a passive form of bacterial translocation that is depended on secreted bacterial surfactants that reduce the agar surface tension, thereby facilitating movement (*7*). Specifically, the expansive forces produced by cell division coupled with reduced surface tension mediate colony spreading. In contrast, motility powered by surface exposed organelles is considered an active form of motility and is identified by the complex pattern formation of motile cells on agar surfaces. Differences in pattern formation are attributed to environmental cues that modulate the behavior of motility organelles, such as the shifts from clockwise to counter clockwise rotation of flagella, which mediates swarming (4). However, a form of active motility that is independent of flagella/ pili has been described in other organisms such as *Myxococus xanthus* (*7*)and *Mycoplasma mobile* (*7*). Evidence for factors that power such motility exist, but no definitive mechanisms have yet been defined (*7*).

Currently no evidence directly supports the idea that FPI motility is an active form of surface translocation; no force generating factors similar to those in *M. xanthus* adventurous (A) motility have been identified. However, it has been observed that the two-component regulatory system GacS/GacA that positively regulates biofilm development and negatively regulates organelle-mediated motility in *P. aeruginosa*, also has a negative affect on FPI motility. Triple mutants of FPI motile cells with a null mutation in *gacA* (*fliC pilA gacA*), exhibit complex colony expansion patterns similar to those produced by swarming *P. aeruginosa.* The ability to promote this behavior by eliminating a regulator suggests that FPI motility may be an active form of motility. Regardless of how FPI motility is mediated, this type of surface translocation appears to play an important role during chronic pulmonary infections, specifically in the seeding dispersal process, when motility organelle gene expression is reduced and rhamnolipid biosynthesis gene expression is increased (*2*, *5*).

Rhamnolipids are a virulence factor that promotes the intracellular invasion of pulmonary epithelial cells by *P. aeruginosa.* The intracellular invasion of pulmonary epithelial cells by *P. aeruginosa* is facilitated by a direct affect of rhamnolipids on the pulmonary epithelium that causes cellular depolarization and compromises the tight-junctions between the cells. The mechanism mediating this rhamnolipid activity is unknown. However, it was demonstrated, that *P. aeruginosa* cells deficient in rhamnolipid biosynthesis were unable invade and the addition of exogenous rhamnolipids was sufficient to restore the invasive behavior(*8*).

Rhamnolipids are a virulence factor produced most efficiently by *P. aeurginosa*. Rhamnolipids are a microbial glycolipid produced primarily and most efficiently by *P. aeurginosa*, a virulence factor associated with chronic pulmonary lung infections, and a bio-surfactant with many potential beneficial applications in the healthcare industry. There are two major types of rhamnolipids, mono- and di-rhamnolipid (*9*). Rhamnolipids are produced in the *P. aeurginosa* cytoplasm by three enzymes, RhlA (required for biosynthesis of the fatty acid rhamnolipid precursor 3-[hydroxyalkanoyloxy] alkanoic acid [HAA]), RhlB (directs the synthesis of mono-rhamnolipids by adding one rhamnose group to HAA) and RhlC (adds a second rhamnose group to mono-rhamnolipids for the synthesis of di-rhamnolipid). Much of the work characterizing the chemical properties of

rhamnolipids has centered on their industrial applications; the surfactant properties of rhamnolipids, particularly di-rhamnolipids, make them ideal candidates for use in bioremediation. Medical applications of di-rhamnolipids are also of interest. A recent study demonstrated that topical application of di-rhamnolipids accelerates the healing time of mice burn wounds. Unfortunately, the use of rhamnolipids is restricted by the costs associated with producing rhamnolipids in a bacterial pathogenic; *P. aeruginosa* is currently the most efficient rhamnolipid producing microbe (*9*). Efforts to engineer a heterologous producer of *P. aeruginosa* mono-rhamnolipids have been reasonably successful, but no bacterial strains able to efficiently produce di-rhamnolipids are available (*9*). Only *P. putid*a is able to synthesize some amount of di-rhamnolipids (*9*, *10*). One can only assume that efforts towards developing strains capable of producing di-rhamnolipids in a heterologous host have been met with unforeseen complications. It is interesting that only another *Pseudomonas* strain has been somewhat successful at the heterologous production of di-rhamnolipids, since *Pseudomonas* species, including *Pseudomonas putida* produce their native rhamnolipids at relatively low levels(*9*). It is likely that there is a common element in *Pseudomonas* strains that allows for the heterologous production di-rhamnolipids.

Glycolipids are amphiphilic molecules that required an active transport for secretion. Two well-characterized glycolipids are lipopolysaccharide (LPS) (*11*) and nodulation factor (*12*), which is a glycolipid produced by *Rhizobium sp.* that promotes the development of nodules on plant roots. Both of these glycolipids use dedicated active transport mechanisms mediated initially by an ABC transport system. ABC transporters in Gram-negative bacteria are located on the inner membrane and mediate transport of a substrate from the periplasm to the cytoplasm (importers) or from the cytoplasm to the periplasm (exporters). An outer membrane complex is required for the final export across the outer membrane(*13*). **To our surprise, to date, there are no reports describing an active transporter involved in rhamnolipid secretion**. *Understanding mechanism by which P. aeruginosa exports rhamnolipids is important for many reasons including that the inability to export synthesized rhamnolipids is likely to be cytotoxic and that constructing strains able to synthesize and export di-rhamnolipids will be useful in the production of biosurfactants for use in the healthcare industry.* The work proposed here is a first step towards understanding the mechanism of rhamnolipid secretion. We envision that the elucidation of the complete export system, especially the outer membrane component of system that mediates rhamnolipid export from the periplasm will serve as potential candidates for the development of novel therapies for the treatment of chronic *P. aeruginosa lung infections.*

Innovation:
Rhamnolipids are important virulence factors in *P. aeruginosa* pathogenesis. Surprisingly the secretion pathway for rhamnolipids has yet to be discovered despite its relevance in both industrial and medical fields. We propose for the first time that rhamnolipids are secreted by the *P. aeruginosa* Gld-like ABC transport system that is related to similar systems known to mediate flagella/pili independent (FPI) motility in other organisms. Using a novel synthetic biology approach to express both *P. aeruginosa* rhamnolipid biosynthesis and *gld*-like ABC transporter genes in *E. coli*, we aim to both define the mechanism of rhamnolipid secretion and increase production for the first time of di-rhamnolipids, a bio-surfactant that has industrial and medical importance. Finally, this project should provide important insights into the mechanstic aspects of this unique motility system that is critical in *P. aeruginosa* pathogenicity.

Impact:
Rhamnolipid secretion plays a key role in sliding motility and is critical in the pathogenesis of *P. aeruginosa* pulmonary infections due to its ability to compromise the host's immune defense system and its activity in dispersal of the biofilm cells. Specifically, dispersal results after the formation of seeding dispersal fruiting bodies and allows the biofilm cells that are facing nutrient deplete environments to escape the biofilm and colonize new areas. Understanding the mechanism of rhamnolipid secretion will impact on a variety of important health-related areas, including the production of an important surfactant virulence factor that directly impacts the host and facilitates pathogenesis and the development of new therapeutics designed to target *P. aeruginosa* in pulmonary-related infections.

Approach

Preliminary Findings: An *in silico* analysis of Pseudomonas aeruginosa open reading PA3669-PA3672 frames reveals genes that code for protiens with high predicted functional orthology to a family of proteins known to mediate FPI motility in other organisms.

Rational: The production of a glycolipid-like substance and FPI motility are intimately linked behaviors. FPI motility in other Gram-negative organisms, such as Legionella pneumonia have been described. L. pneumonia FPI motility is known to depend on the secretion of an surfactant of unknown kind. It was recently discovered that the secretion of the surfactant is requires the presence of an inner membrane bound ABC transporter and TolC (an outer membrane secretin). A data mining approach ?(methodically clicking on every ORF on the pseudomonas genome database until the first Gld-like gene was found)? Was taken in attempting to find *P. aeruginosa* ABC transporter proteins orthologous to the L. pneumonia surfactant secretion system. Functional orthologs were not identified but an unrelated family of proteins, best characterized in Flavobacterium johnsoniae was discovered. A total of 12 genes were predicted to be functional orthologs to a the Gld system component (Data not shown). Of particular interest was a four gene cluster predicted to code for a Gld-like ABC transporter (Fig 2). The primary amino acid sequence for each gene was further analyzed using HH-PRED (X) and it was determined that two of the proteins (PA3670 and PA3671) were predicted to be functional orthologs to the transmembrane domain proteins of the ABC transport system that mediates the secretion of nodulation factor in Rhizobium species. PA3672 is predicted to be the ATP binding protein component of the complex. A fourth protein, PA3669 is of unknown function (DUF4340). We speculate that this protein, is somehow involved in the function of the ABC transporter for two reasons: 1) All four genes are predicted to transcribe from the same promoter; 2) There are several other examples of genes with domain of unknown function 4340 coded from a predicted four gene operon with genes predicted to code for an complete ABC transporter (data not shown).

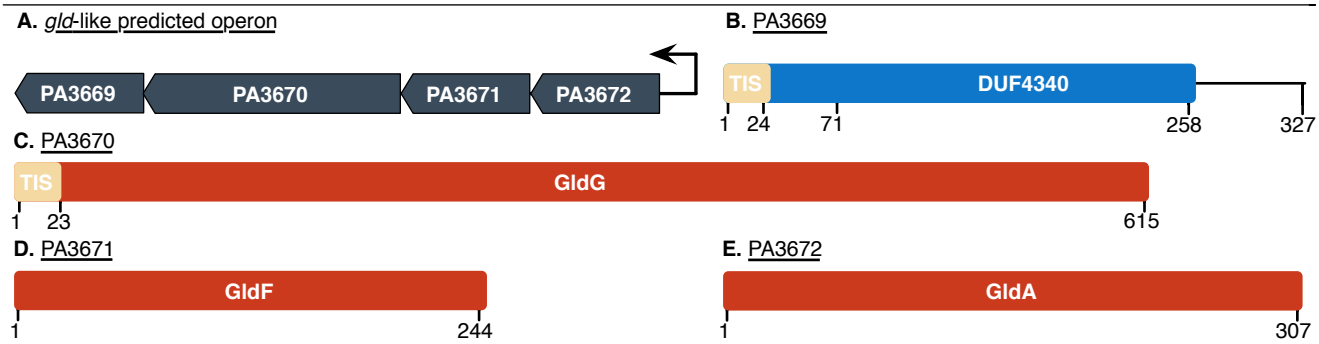

Figure 2. *In silico* predictions of the Gld-like gene cluster and protein functional domains. A) The gld-like gene cluster is predicted to be coded on a operon. B) PA3669 codes for a protein with a domain of unknown function. C) PA3670 orthologous to GldG. D) PA3671 orthologous to GldF and NodJ. E) PA3672 orthologous to GldA and NodI. Initial analysis and predictions about gene cluster organization and TIS was performed on the Pseudomonas genome database. Primary amino acid sequence analysis was performed using the HHpred homology detection and structure prediction tool found on the Max-Planck Institute for Developmental Biology website.

PA01 ORF	Domain of interest	Function	Prob.	E-value	P-value
PA3669	PRK10893	PLEX: Periplasmic Lipooligosaccharide export	91.5	2.5	1.7E-5
	PF14328	DUF4340: conserved in species with PFI motility.	100	1.3E-27	9.1E-33
PA3670	TIGR03521	GldG: putativeABC trans. component associated with gliding motility	100	2.9E-85	2E-90
PA3671	TIGR03518	GldF: putative ABC trans. permease associated with gliding motility	100	5.4E-24	3.6E-29
	TIGR01291	NodJ: ABC trans. perm ease required for nodulation factor export	100	3E-24	2E-29
PA3672	TIGR03522	GldA: ABC trans. ATP binding protein associated with gld motility	100	1.1E-63	7.6E-69
	PRK13537	NodI: ABC trans.r ATP binding protein required for nodulation factor export	100	1..2E-61	9.3E-67

Table 1. Primary amino acid sequence results for each Gld-like protein. Domains of interest are listed with statistical values for each. Complete results can be accessed on http://toolkit.tuebingen.mpg.de/hhpred with the following JobID numbers: PA36782-6881199, PA3671-3485792, PA3670-7112218, PA3669-2991312.

Aim 1: Define the involvement of the Gld-like putative ABC transport system coded by the uncharacterized loci PA3669-PA3670-PA3671-PA3672 on FPI surface translocation and rhamnolipid secretion by mutational analysis. *Hypothesis: FPI surface translocation in PA is dependent on the secretion of rhamnolipids by the PA Gld-like ABC transporter, a system known to mediate FPI motility in other bacterial species.*

Rational: The hypothesis to be tested in this aim is based on the above mentioned background information and preliminary findings. The proposed analysis will examine the postulated relationship between *P.*

aeruginosa FPI motility and the conserved *gld*-like gene family. The putative PA Gld-like ABC transporter is proposed to mediate FPI motility by secreting rhamnolipids, a substance required for FPI motility. Three of the Gld-like proteins (PA3670-PA3672) are predicted to form an ABC transport complex. The role PA3669 playes in rhamnolipid secretion is unknown, but it is predicted to be involved in the ABC transport function. Rather than speculating on the possible function of PA3669, we will determine if it is involved by examining the effect of its absence on FPI motility and RHL secretion. ABC transporters require the presence of all protein components in order to form a functional transport complex. Deletion of any gene should result in the abolishment of the RHL secretion in a wild-type background and FPI motility in a *ΔfliC pilA* background.

Aim1.1: Determine if the PA *gld*-like genes are required for FPI surface translocation. *Hypothesis: Null mutations in any one gld-like gene will result in the lack of FPI motility inΔfliC pilA mutant strains.*
Experimental design: This sub-aim is designed to address the hypothesis that a deficient Gld-like ABC transporter will abolish FPI surface translocation. Phenotypic analysis of FPI in PA strains with targeted deletions to each *gld*-like gene will be conducted. It is expected that mutations in any single *gld*-like gene will result in a loss of FPI motility. All four *gld*-like genes will be independently tested to determine if they are all required for FPI surface translocation. Genetic complementation analysis will confirm that the loss of FPI motility in *gld*-like deficient strains is due to the loss of the *gld*-like gene.

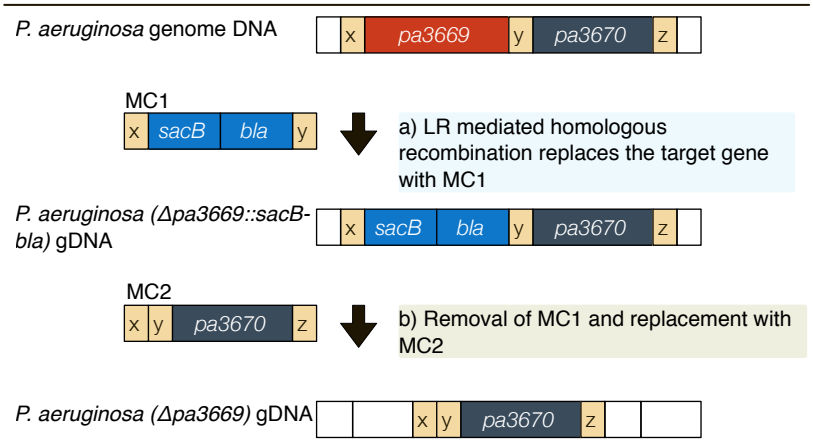

Fig 3. Mutagenesis of *pa3669*. (a) MC1, composed of sacB-bla selection markers flanked with homologous ends (regions x and y) will be introduced into PA/pRKaraRed cells. LR genes will be induced, prompting the homologous recombination between MC1 and pa3669. (b) MC2 with homologous flanking ends to the 5' end of MC1 and a DNA sequence down stream of the 3' end of MC1 (regions x and z) will be introduced into recombinant cells. LR genes will be induced prompting the replacement of MC1 with MC2.

Creation of mutant strains by homologous recombination: A two-step Lambda Red (LR) mutagenesis process that results in in-frame gene deletions will be used. This method was modified by (x) specifically for mutagenesis in PA and all mutagenesis vectors will be requested from this group. The system consist of three elements: 1) a LR plasmid (pRKaraRed) that contains the genes necessary to mediate homologous recombination (*gam, bet, exo*) under the control of an arabinose inducible promoter and a selectable marker); 2) a mutagenesis gene cassette (MC1), linear DNA composed of selection markers flanked by short sequences homologous to the target gene; 3) a second cassette (MC2) that removes the selection markers. All exogenous DNA will be introduced into PA by electroporation. Cells carrying pRKaraRed will be selected by screening for tetracycline resistance. Cell that underwent successful recombination with MC1 are selected by screening for sensitivity to sucrose and resistance to ampicillin. Cells that underwent successful recombination with MC2 will be selected by screening for sensitivity to ampicillin and resistance to sucrose. All mutations will be verified by sequencing a region surrounding the site of mutagenesis. Figure 1A illustrates the process.

Analysis of FPI motility: *P. aeruginosa Δ fliC pilA* mutants have been shown to exhibit sliding motility on 0.5% agar surfaces that promote swarming motility. Strains containing deletions of the *gld*-like genes in a *Δ fliC pilA* mutant background will be screened for the lack of FPI motility. FPI surface translocation analysis of *Δ fliC pilA pa3669, Δ fliC pilA pa3670, Δ fliC pilA pa3671,* and *Δ fliC pilA pa3672* strains will be conducted as describe below (REF) in order to confirm that all genes are required for FPI motility. To perform the FPI motility assay, mid-log phase liquid cultures will be grown and 10 ul corresponding to 5 X 10^6 cells will be placed on M8 minimal medium 0.5% agar plates. The plates will be incubated overnight at 30°C followed by a 24-hour incubation period at room temperature. Whole colonies will be imaged using a stereomicroscope. The degree of surface translocation will be determined by measuring the extent of colony expansion from the point of inoculation and the development of complex colony morphology. The presence or lack of FPI surface motility in strains lacking *gld*-like genes will be determined by comparing their motility phenotypes with control strains: *Δ*

fliC pilA (positive for FPI surface motility) and *Δ (pos* (negative FPI motility control, also rhamnolipid biosynthesis deficient) (Figure 1). FPI surface translocation is characterized by a colony with significant expansion from the point of inoculation with minor colony morphology complexity (Fig. 2B). Strains with a deficient *gld*-like gene that exhibit this type of colony morphology will be considered as positive for FPI motility. In order to determine if *gld*-like gene deficiencies are specific to surface translocation, deletions to the above mentioned *gld*-like genes will be generated in wild-type PA and mutant strains will by analyzed for swimming motility. Flagella-mediated swimming motility is not affected by deficiencies in rhamnolipid biosynthesis and thus should not be affected by deficiencies in RHL export. Swimming motility analysis will be conducted by placing 10 ul of cells on LB 0.3% agar plates and incubating at 37°C for 24 hrs. These plates will be imaged and the expansion zone from the inoculation origin will be measured and compared to *Δ fliC* (deficient in swimming motility). All motility experiments will be conducted in triplicate at least three separate times.

Genetic complementation analysis: One plasmid containing a wild-type alleles and promoter of the four *gld*-like genes will be constructed in a pUCP plasmid using standard recombinant DNA technology. The pUCP plasmid family is derived from pUC18/19 and designed to be stably maintained in PA and *E. coli.* The plasmids will be constructed in *E. coli* to facilitate genetic manipulation and then transferred by electroporation into the appropriate PA strains. Restoration of FPI surface motility in strains deficient in each *gld*-like gene will confirm that the lack of the *gld*-like gene is attributed to loss of FPI mediated surface translocation. An empty pUCP vector will also be introduced into null mutants in order to verify that a restoration of FPI motility is not induced by the presence of the vector elements. FPI surface translocation in complemented mutants will be analyzed as described above.

Expected Results: We predicted that the loss in any one *gld*-like gene will result in the abolishment of FPI surface translocation in the *ΔfliC pilA* mutant background. Deficient FPI motility should not be attributed to a general growth defect. We anticipate that growth rate analysis results will indicated that the growth rates of the mutants will be indistinguishable from wild-type cells. This will confirm the FPI motility deficiency is not due to a growth defect. The *gld*-like gene deficiencies should not result in a general lack of motility. Wild-type PA with null mutations in any one of the *gld*-like gene should exhibit swimming motility comparable to the parental wild-type strain. Strains deficient in any of the four *gld*-like genes that lack FPI motility should exhibit a restoration of FPI motility when a complementation plasmid is introduced and expression of the empty vector should not restore FPI surface motility.

Pitfalls and Resolutions: It is possible that the triple mutants containing null mutations in any of the *gld-like genes* in *Δ fliC pilA* mutant background may result in either reduced FPI motility or sustained FPI motility compared to the parental strain. This may be explained the possibility of genetic redundancy of the ABC transporter. Interestingly, a second four-gene cluster predicted to code for a Gld-like ABC transport system was identified in the PA chromosome (PA4036-PA4040). The *gld*-like gene cluster coded by PA3669-3672 is the primary candidate for this study because of previous evidence that the gene cluster is expressed in PA and involved in motility (X). Reduced or sustained FPI motility in triple mutants containing null mutations in the PA3669-PA3670 gene cluster could being suppressed by this or another ABC transporter system; it is possible that this is the correct ABC transporter.

It is possible that PA developed genetic redundancy to avoid RHL induced stress or death resulting from RHL the antimicrobial properties associated with their surfactant qualities that intracellular accumulation could cause. Genetic redundancy may also explain why the RHL secretion pathway has remained elusive. Analysis of the second (PA4036-PA4040) Gld-like ABC transporter would be conducted as described above to investigate if it and not first (PA3669-PA3672) ABC transporter is involved in FPI motility. In addition double null mutants in both ABC transport systems would also be analyzed. The predicted results from this analysis are that FPI motility will be abolished in cells lacking both Gld-like ABC transport systems.

1.2: Determine if the genes that code for the Gld-like ABC transporter are required for RHL secretion motility.

Experimental design: This sub-aim is designed to test the hypothesis that a null mutation in any one gene that codes for the Gld-like ABC transport system will abolish RHL secretion. Null mutants in the *gld*-like genes generated in a wild-type PA background in aim 1.1 will be analyzed for total RHL secretion. Deficiencies in

rhamnolipid export should result in rhamnolipid accumulation within the cells. Thus, total rhamnolipids trapped within PA cells will also be analyzed. Complementation analysis will be conducted with a plasmid that expresses the wild-type *gld*-like genes cluster from its native promoter

Analysis of rhamnolipid secretion: To determine the involvement of the *gld*-like genes in rhamnolipid secretion, total rhamnolipids will be measured in cell-free supernatant of *gld*-like mutants generated in a wild-type background. The orcinol method (19) will be employed in this analysis. The orcinol method measures the amount of rhamnolipids isolated from cell-free supernatant using spectroscopy. Rhamnolipids from spent medium supernatant are extracted with diethyl ether and samples are rehydrated in water and treated with sulfuric acid. The acid treatment hydrolyzes the rhamnose groups converting them into methyl furfural with which orcinol reacts producing a blue-green absorbing molecule. The amount of blue-green absorbance is concentration dependent. A standard curve will be generated with known amounts of purified rhamnolipid (Sigma) in order to quantitate the amount of rhamnolipids contained in the cell free supernatant. The RHL content of the cell-free supernatant of strains with null mutations in each of the *gld*-like genes will be compared to RHL contained in wild-type cell supernatants. Controls will include a *Δ. C strain deficient in rhamnolipid biosynthesis and a standard laboratory strain of E. coli (naturally deficient in rhamnolipid secretion) in order to ensure that measurements using this assay can be truly attributed rhamnolipids secreted from a cell.*

Analysis of intracellular rhamnolipids: Rhamnolipids are synthesized within the cytoplasm, thus deficiencies in the Gld-like ABC transporter, would be expected to result in the intracellular accumulation of rhamnolipids. Thus null *gld*-like mutants in a wild-type will be analyzed for the presence intracellular rhamnolipids. Rhamnolipid biosynthesis is quorum sensing regulated (REF). Liquid cultures of wild-type and *gld*-like mutants PA will be grown to late stationary phase for intracellular rhamnolipid analysis. After reaching late stationary phase, the cells will be pelleted and the supernatant will be removed. A cell lysis solution will be added to pelleted cells and rhamnolipids will be extracted from the lysate as described above. Total intracellular rhamnolipids will be measured using the orcinol method. Analysis of wild-type cells will serve as a positive control for the transient presence of intracellular rhamnolipids and Δ *rhl* mutants will serve as a negative control for the absence of intracellular rhamnolipids.

Genetic complementation analysis: The gld-like gene cluster complementation plasmid will be used to complement gld-nul mutants. The same approach as listed above will be used.

Anticipated results: It is predicted that null mutations to any one *gld*-like gene will result in an abolishment of rhamnolipid secretion. Analysis of extracellular rhamnolipids of strains containing *gld*-like gene null mutations should result in no detectable extracellular rhamnolipids. Analysis of *Δ nal mutants* should result in no detectable extracellular rhamnolipids. Analysis of intracellular rhamnolipids in the *gld*-like gene null mutants should result in an intracellular accumulation of rhamnolipids. Analysis of wild-type cells should result in detectable intracellular rhamnolipids. It is predicted that minimal intracellular rhamnolipids will be detectable since this strain is capable of secreting rhamnolipids. An analysis of this strain will establish the total amount of rhamnolipids natively found within a cell. Analysis of the *Δ f t strain should exhibit no intracellular rhamnolipids since this strain is completely deficient in rhamnolipid biosynthesis.* Complementation analysis of the gld-like genes with an empty vector control should result in the absence of detectable extracellular rhamnolipids. Introduction of the complementation plasmid in the gld-like null mutant should result in restored detection of extracellular rhamnolipids.

Pitfalls and resolutions: The major problem that could be encountered in the experiments proposed in this aim would be if the *gld*-like gene null mutations do not result in a loss of rhamnolipid secretion. PA synthesizes two main types of rhamnolipids, mono- and di- rhamnolipid. There is some indication that a non-specific pathway could mediate the secrete mono-rhamnolipids. Attempts at heterologous production of rhamnolipids in *E. coli* for industrial purposes have only resulted in the successful production of mono-rhamnolipids. However, the robust surfactant properties of di-rhamnolipids make them an ideal candidate for use in bio-remediation applications by industry. We speculated that the lack of reports describing the production of di-rhamnolipids in *E. coli* suggest that an unknown problems may be preventing progress in this area. Since *E. coli* is capable of secreting mono-rhamnolipids by an unknown pathway, possibly by an ABC transporter that mediates the secretion of amphiphilic substance, extracellular rhamnolipids in *gld*-like null mutant strains could consist exclusive of mono-rhamnolipids. Should this hypothetical problem arise, the following alternative

hypothesis will be tested: the PA Gld-like ABC transporter mediates the secretion of di-rhamnolipids. Analyzing the types of rhamnolipid species secreted by strains with null mutations in the *gld*-like ABC transporter genes will test this hypothesis. Rhamnolipids species will be extracted as described above and quantitated by high pressure liquid chromatrography-mass spec (HPLC-MS). This technique was previously employed by (x) to characterize the various rhamnolipid species secreted by wild-type PA. Rhamnolipids will be purified from cell free supertantant of the various mutants described above. The total amount of each rhamnolipid species will be quantitated. The following results are expected: the supernatant of the *gld*-lke gene null mutants are expected to contain only mono-rhamnolipids and be devoid of di-rhamnolipids. If the alternative hypothesis is investigated the work performed in this aim will still support the overarching hypothesis.

Aim2: Determine if the PA Gld-like ABC transporter system can mediate the secretion of rhamnolipids in *E. coli*. *Hypothesis: Expression of the Gld-like ABC transport system in recombinant RHL producing E. coli will increase the efficiency of rhamnolipid production by providing a pathway for rhamnolipid secretion.*
Rational: Optimizing the production of rhamnolipids in non-pathogenic bacteria is of great interest to groups using bio-surfactants for bioremediation applications. Efforts to engineer *E. coli* for the production of rhamnolipids have proven difficult. To date, only the production of mono-rhamnolipid has been reported (REF), and the efficiency of its production in *E. coli* is too low for industrial use (REF). *P. aeruginosa* remains the preferred organism for industrial production of rhamnolipids due to its high levels of native production of di-rhamnolipids, which is the most active form of the surfactant. However, the development of a heterologous system in *E. coli* for rhamnolipid production would still be useful to increase surfactant yields and improve safety during the production process by using non-pathogenic organism. Di-rhamnolipids are also of interest for their use in the healthcare industry for the treatment of burn wounds and for use in cosmetics. The secretion of rhamnolipids through an active secretion pathway is required because the amphiphilic properties of the substance prohibit passive export pathways. The ability to detect mono-rhamnolipids produced in *E. coli* could be attributed to either released rhamnolipids from lysed cells or secretion of mono-rhamnolipids by a non-specific active export mechanism. Thus, the studies proposed in this aim will determine if the transcriptional expression of the *P. aeruginosa* Gld-like ABC transport system, hypothesized to mediate the secretion of rhamnolipids, increases the amount of extracellular mono and di-rhamnolipids produced by *E. coli*. This aim will also serve to test the overarching hypothesis of this proposal by determining if the proteins coded by the *P. aeruginosa gld*-like genes mediate the secretion of rhamnolipids by correlating the presence or absence of the ATP binding component of the ABC-transporter with the rhamnolipid secretion phenotype of *P. aeruginosa* rhamnolipid producing recombinant *E. coli*.

Aim 2.1 Construct a strain of *E. coli* capable of producing mono- and di- rhamnolipids and evaluate cell viability, and the synthesis and location of rhamnolipid species. *Hypothesis: A rhamnolipid producing strain of E. coli will exhibit reduced cell viability caused by the accumulation of intracellular di-rhamnolipids.*
The hypothesis for this aim was developed based on the background information presented above, specifically the following points should be considered: Important points to consider are the following: 1) Only the production of mono-rhamnolipids has been reported in *E. coli* and to our knowledge there are no published reports attempting to produce di-rhamnolipids in *E. coli,* 2) Rhamnolipids similar to all other microbial glycolipids require a secretion pathway for extracellular transport, 3) Rhamnolipids are a bio-surfactant with antimicrobial properties, and 4) Di-rhamnolipids have the most robust surfactant properties. The work proposed here will aim support the hypothesis for this aim by confirming that mono-rhamnolipids and di-rhamnolipid **intracellular** concentrations are high in bacterial cells that do not have an active rhamnolipid secretion pathway.

Experimental Approach: The goal of this aim is to construct a strain of *E. coli* that is capable of producing both mono- and di-rhamnolipids and to test the intracellular and extracellular localization of the two main rhamnolipid species. The viability of cells capable of producing only mono-rhamnolipids or both mono- and di-rhamnolipids will also be tested to determine if the intracellular accumulation of di-rhamnolipids in the absence of a dedicated secretion pathway results in reduced cell viability. We anticipate that the production of both mono- and di-rhamnolipids will result in reduced cell viability, but that the viability of strains producing both rhamnolipid species will be more highly compromised when compared to strains that only produce mono-rhamnolipids.
Construction of a rhamnolipid producing strain: Two expression plasmids will be generated. The first

plasmid will harbor the genes required in the first two steps of rhamnolipid biosynthesis, rhlA (the first enzyme in the pathway) and rhlB (required for mono-rhamnolipid biosynthesis) will be controlled by a common inducible promoter. A distinct inducible promoter will control the expression of rhlC (required for the biosynthesis of di-rhamnolipids) on the second plasmid. Cells induced for the expression of rhlA-rhlB gene cluster alone will be analyzed for the affects that mono-rhamnolipids have on cell viability. Cells induced to express rhlA-rhlB and rhlC will be analyzed for the effects that the presence of di-rhamnolipids have on cell viability. Expression rates will be modulated in order to determine if increasing the expression of the rhamnolipid biosynthesis genes is correlated an increased level of mono- and di-rhamnolipids.The intracellularand extracellular amounts of mono-rhamnolipids and di-rhamnolipids will be measured in *rhlA-rhlB* induced cells and in *rhlA-rhlB;rhlC* induced cells as described in aim 1.2.

Construction of the rhamnolipid producing strain: A four gene construct composed of the an artificial *rhlA-rhlB* operon under the control of an ITPG inducible promoter, rhlC under the control of an anhydrotetracycline inducible promoter and a antibiotic resistance marker will be generated using standard recombinant DNA methods. The rhlA-rhlB synthetic operon will be controlled by ^{P}LlacO-1 and rhlC regulated by ^{P}LtetO-1.The two promoters that will be used for this study developed by (x) are designed for the quantitative modulation of gene expression in E. coli (DH5 α . colStudies characterizing this class of promoters found that in the absence of the inducer, transcript levels are correlated with the plasmid copy number and that gene induction can be tightly regulated with an induction range of up to 5000 fold. Construction of the four-gene cluster to be inserted into the DH5 α H5echromosome is depicted in figure X. The inducible four-gene construct will be generated with flanking ends homologous to the chromosomal insertion site; chromosomal insertion will be performed by Lambda Red mediated homologous recombination as described in the first aim.

Characterizing rhamnolipid gene expression from the regulatable promoters: In order to confirm that the inducible promoters can modulate the expression of the rhamnolipid in an inducer dependent manner, reverse transcriptase quantitative PCR (RTqPCR) will be used to measure gene expression levels under increasing levels of induction. Cells will be grown in liquid culture with either no inducer or increasing levels of inducer. Mid-log cells will be collected and RNA will be collected using a standard commercial Qiagen RNA isolation kit. RTqPCR will be conducted using a standard SYBR Green one step RTqPCR kit. Standard house keeping genes with known expression will also be monitored as controls in order to ensure the fidelity of expression analysis conducted on the rhamnolipid biosynthesis genes. mesurements monitored during Gene expression analysis will be conducted in triplicates three different times to determine the reproducibility and the variation that should be expected.

Growth rate analysis of rhamnolipid producing *E. coli*: The affects that mono- and di- rhamnolipids have on growth rate will be determined. Experiments will be conducted that compare growth rates of wild-type DH5 α H5 cells with uninduced and induced rhamnolipid-producing DH5 α H5duFirst, cells expressing the genes necessary for mono-rhamnolipids will be analyzed by growing cells in the presence of increasing concentrations of IPTG. Cells expressing *rhlC* (di-rhamnolipid) will be grown in the presence increasing concentrations of anhydrotetracycline. Growth curve analysis will be compared to uninduced cells and wild type cells. All growth curve analysis will be conducted by growing cells in a 96 well plate format and automated optical density readings will be taken by using a plate reader.

Analysis of inter/extra cellular rhamnolipids: The localization of mono- and di-rhamnolipids will be measured in the rhamnolipid producing strain of *E. coli*. Analysis of mono-rhamnolipid expressing cells will be conducted by first measuring the total amount of mono and di-rhamnolipid in the cell-free supernatant. Cells will be grown in liquid culture in the absence and presence of increasing levels of the inducer. Samples of growing cultures will be collected at mid-exponential phase. Cells will be pelleted from the supernatant and resuspended in fresh growth medium lacking the inducer. A small fraction of resuspended cells will be used for plating assay to determine the number of viable cells present in the culture. The remaining cells will be used to determine the intracellular rhamnolipid concentration. Analysis of cells expressing mono and di-rhamnolipds will be conducted as stated above. Rhamnolipids will be extracted as described above and HPLC-MS will be used for quantitating the total amounts of each rhamnolipid species as described in aim 1.

Expected result, Pitfalls, and Resolutions: *E. coli* cells expressing the genes required for mono-rhamnolipid biosynthesis are expected to experience moderate growth defects that are dependent on the level of gene induction when compared to wild-type and uninduced rhamnolipid producing *E. coli*. Growth curve analysis will show that uninduced cells will have a growth rate not significantly different from wild-type cells. Induction that results in minimum induction will result in a minimal retardation in growth. A maximum induction level that renders cell unviable is expected. Cells expressing di-rhamnolipids will have a more sever retardation in growth with a much lower maximum induction level. Analysis of inter/extra cellular rhamnolids of cells only expressing mono-rhamnolipids will reveal that cell-free supernatant exclusively contains mono-rhamnolipid. Intracellular mesurements will reveal that, like the cell free supernatant, the cytosol exclusively contains mono-rhamnolipids. Inter and extracellular concentrations of rhamnolipids will be directly correlated with the degree of expression produced by specific concentrations of inducer present in the growth medium. The cell-free supernatant of *E. coli* expressing di-rhamnolipid will be composed primarily of mono-rhamnolipids. The detection of small amounts of extracellular di-rhamnolipids is expected but is likely due to the release rhamnolipids from dead cells that experienced a high degree of accumulated intercellular rhamnolipids. Intercellular concentrations of di-rhamnolipids will be significantly higher than mono-rhamnolipids and will be directly correlated with the degree of *rhlC* gene induction. Reduced cell viability at higher states of induced rhamnolipid production result in the accumulation of intercellular rhamnolipids. Taken together the expected results will demonstrate that inefficient rhamnolipid production in *E. coli* is due to the intercellular accumulation of rhamnolipids. Retarded growth and eventual cell lyses are likely due to excessive detergent induced stress caused by an inability to export rhamnolipids, most importantly di-rhamnolipids. One pitfall that can be associated is that the promoters being used for these studies express too highly, making it difficult to culture viable rhamnolipid producing strains. Different promoters will be used to compensate for such a pitfall.

Aim 2.2 Determine the effect of the production in *E. coli* of the PA Gld-like ABC transporter on the efficiency of synthesis and secretion of mono-rhamnolipid and di-rhamnolipids. *Hypothesis: The production in E. coli of the PA Gld-like ABC transporter will increase the efficiency of mono-rhamnolipid production and allow for the production and secretion of di-rhamnolipids.*

Analysis of intracellular and extracellular rhamnolipid composition of *E. coli* cells producing rhamnolipids and expressing the PA *gld*-like genes. The PA *gld*-like gene under control of a constitutive promoter will be introduced in to the chromosome of *E. coli* at the lamba attachment site that are expressing the mono- and di- rhamnolipid synthesis genes and the intracellular and extracellular localization of mono- and di- rhamnolipids will be tested as described above. As a negative control the strain without the PA *gld*-like genes will be examined.

Anticipated results: Rhamnolipid secretion is expected to be conferred in cells expressing the PA *gld*-like gene expression construct. Taken together, these data demonstrates that the ATP binding protein component of the Gld-like ABC transporter is required for rhamnolipid secretion. Analysis of rhamnolipid secretion in *E. coli* expressing the rhamnolipid biosynthesis genes will reveal if the expression of the PA *gld*-like gene expression construct confers *E. coli* the ability to secreted di-rhamnolipids and increases the efficiency of mono-rhamnolipid secretion. High levels of rhamnolipid gene induction will result in higher levels of extracellular rhamnolipids when compared to low and non-induced conditions. Intracellular rhamnolipids will no be significantly present. qPCR will be used to determine if the PA *gld*-like genes are expressed in the *E. coli*.

Pitfalls and Resolutions: If the there are not differences in rhamnolipid composition and localization between the E. coli strains expressing the PA *gld*-like gene expression construct and the strain not expressing this construct, is possible that the Gld-like ABC transport system is not expressed under these conditions. If expressing the Gld-like ABC transporter is not successful a completely different approach will be taken to demonstrate that the ABC transporter complex mediates the transport of rhamnolipids across the lipid bilayer. An in vitro approach will be to conduct substrate translocation assays using liposomes reconstituted with purified ABC transporter complex.

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